Chapter 3

Programming of intestinal cholesterol absorption in mice: sensitive window of ezetimibe treatment

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Abstract

SCOPE: Maternal administration of the cholesterol absorption inhibiting drug ezetimibe during the lactation period reduces cholesterol absorption in the offspring up into adulthood via epigenetic programming. Developmental programming of metabolic processes is known to have sensitive windows. We now determined whether the sensitive window, in which ezetimibe programs cholesterol absorption, extends beyond the lactation period.

METHODS AND RESULTS: We administered ezetimibe to C57BL6/JOlaHsd mice via the food (0.005 w/w %) for three weeks after weaning and compared these mice to non-treated mice. Cholesterol absorption was measured during and at six weeks after ezetimibe treatment using dual-isotope methodology. Feces was collected during, and at two and six weeks after ezetimibe administration to determine sterol excretion.
During its administration, ezetimibe decreased cholesterol absorption (-78 %, $p = 0.004$) and increased fecal NS excretion (+ 301%, $p = 0.004$), compared to controls. Two and six weeks after ezetimibe administration, however, fecal NS excretion was similar to the control group. At 6 weeks after ezetimibe administration, neither cholesterol absorption differed significantly from control mice ($p = 0.33$), nor did plasma cholesterol levels, lipoprotein profiles, or intestinal NPC1L1 mRNA expression.

CONCLUSIONS: This study demonstrates that in mice the sensitive window for ezetimibe-induced programming of cholesterol absorption does not extend beyond the lactation period.

Introduction

Cholesterol absorption is related to both cholesterol gallstone disease[160] and atherosclerotic cardiovascular disease (CVD)[161]. These conditions are responsible for substantial morbidity and, in case of CVD, also mortality[162]. Higher cholesterol absorption results in hypercholesterolemia[163], which is a well-established risk factor for atherosclerosis development[164]. Cholesterol is absorbed by the enterocytes of the small intestine via the Niemann-Pick C1-Like1 (NPC1L1) protein[54]. Intestinal cholesterol absorption can be attenuated by inhibition of NPC1L1 function, for example by an inactivating mutation[165] or the drug ezetimibe[161, 163]. Inhibiting cholesterol absorption with ezetimibe[50] reduces hypercholesterolemia[163], increases fecal neutral sterol (NS) excretion, decreases plasma cholesterol levels[166] and induces an overall improved plasma lipoprotein profile[161]. The clinical relevance of ezetimibe's mechanism has recently been illustrated by the IMPROVE-IT trial, where adding ezetimibe to statin therapy resulted in incremental LDL cholesterol lowering and consequently a further significant CVD risk reduction in a secondary prevention setting[167]. These observations emphasize the metabolic impact of cholesterol absorption, its potency to be inhibited by ezetimibe and its association with metabolic health.

Bile is responsible for the micellization of lipids, such as cholesterol, to make them accessible for absorption. The hydrophobicity of the BA pool positively correlates with cholesterol absorption and is determined by the individual bile acid species[168]. Alpha- and β-muricholic acids are considerably hydrophilic and potent inhibitors of cholesterol absorption as well as stimulators of BA synthesis[94, 168, 169]. Disposal of cholesterol occurs quantitatively via fecal excretion of cholesterol or bile acids (BA), after hepatic conversion of cholesterol to BA[170].

Cholesterol levels in breast milk are high, relative to those in infant formula[26]. The high breast milk cholesterol concentration has been suggested to have a lasting beneficial impact on cholesterol homeostasis of the offspring[26, 120]. This phenomenon is consistent with the so called Barker hypothesis, which states that the *in utero* and infant environment permanently changes the development and function of the body, thereby 'programming' adult disease risk[121]. Whether the epidemiologically beneficial effects of breast milk are solely attributable to the substantial differences in cholesterol content between breast milk and infant formula or if other factors are in play is not exactly known.

Recently, we demonstrated that in mice the lactation period is a sensitive window to program intestinal cholesterol absorption of the offspring. Decreasing the dietary cholesterol availability during lactation by exposing the offspring to ezetimibe via the maternal milk programmed a decreased cholesterol absorption

in adulthood via epigenetic reduction of intestinal *Npc1l1* expression[59]. This observation demonstrated the ability of the murine intestine to sense nutritional conditions in early life and to retain an active long-term metabolic memory. Noah *et al.* (2011)[171] postulated that intestinal development in mice is completed at weaning and that substantial epigenetic changes are only likely to occur during this period. On the other hand, Pacha *et al.* (2000)[172] concluded that intestinal development extends beyond weaning, adapting to the change of milk to solid food intake post-weaning. Therefore regulation of these metabolic processes up into adulthood might not only be affected by environmental signals during lactation but possibly also when present during the post-lactation period. An extended sensitive window beyond lactation would indicate an extended period in which strategies can be imposed to beneficially manipulate long-term cholesterol absorption and, indirectly, cardiovascular health.

In the present study we set out to determine whether the sensitive window to program long-term cholesterol absorption extends beyond lactation. We exposed murine male offspring to the drug ezetimibe via solid, cholesterol-containing food during three weeks after weaning. During ezetimibe exposure and six weeks later, possible changes in dietary cholesterol absorption, as well as other cholesterol and bile acid homeostasis parameters were determined to evaluate the sensitive window of ezetimibe-induced programming of cholesterol absorption.

Results

Direct effect of ezetimibe on cholesterol absorption

Figure 1A shows the experimental setup of the study. Throughout the study, the body weight was similar between the ezetimibe-treatment and control group (**Fig. 1B**). At the age of 6 weeks, after three weeks of control or ezetimibe diet, fat and lean mass did not differ between the two groups (**Fig. 1C**). Six weeks after ezetimibe exposure, daily food intake, liver weight, intestinal length, and epididymal fat were not affected by the prior ezetimibe treatment (data not shown). At 2-3 weeks after weaning, cholesterol absorption was profoundly lower in the ezetimibe group compared to controls (-78%, $p = 0.004$; **Fig. 1D**). In agreement with lower cholesterol absorption, the excretion of NS was higher in the ezetimibe group (+301%, $p = 0.004$; **Fig. 1E**).

Ezetimibe treatment lowered fecal BA excretion compared to the control group (-68 %, $p = 0.004$; **Fig. 1F**). Fecal BA composition analysis demonstrates that lower fecal BA excretion is predominantly due to lower αMCA and βMCA excretion in the ezetimibe-receiving mice (**Fig. 1G**).

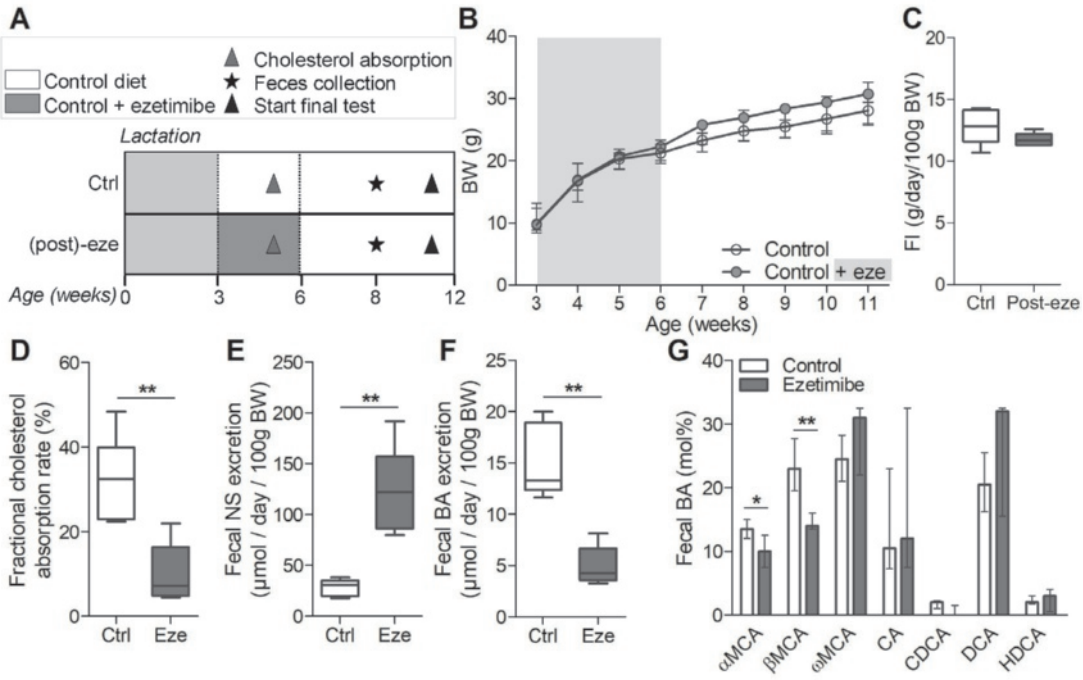

Figure 1: Cholesterol and bile acids during the ezetimibe diet. (A) Simplified schematic of the experimental setup. **(B)** Body weight development (median + range). At the age of 5-6 weeks during ezetimibe treatment: **(C)** Fat and lean body mass, **(D)** Fractional cholesterol absorption, **(E)** Fecal NS excretion and **(F)** Fecal bile acid excretion. **(G)** Fecal BA composition at five weeks age.

Post-treatment effects of ezetimibe on cholesterol absorption and on fecal cholesterol and BA excretion

In our previous study on ezetimibe treatment during the lactation period, the cholesterol absorption in the offspring remained lower, up to 21 weeks after the ezetimibe treatment [59]. In the present experiment, treatment with ezetimibe during 3 weeks post-lactation, did not affect fecal NS or fecal BA excretion at 2 weeks after the last ezetimibe administration (**Fig. 2**), indicating that the sensitive window to program cholesterol absorption does not extend beyond lactation.

At age 11-12 weeks, 5-6 weeks after the last ezetimibe intake, the fractional cholesterol absorption was similar in previously ezetimibe-treated and control mice (**Fig. 3A**). However, a trend for increased fecal total NS excretion was seen in the post-eze group (**Fig. 3B**, p = 0.052). Further compositional analysis of fecal neutral sterols, indicated that fecal cholesterol excretion was significantly higher in the post-eze mice (+37 %, p = 0.0087, data not shown) while coprostanol and dihydroxycholesterol did not differ. Plasma cholesterol was similar between the

groups (**Fig. 3C**), as well as lipoprotein distribution (data not shown). Biliary cholesterol was not significantly different between control and post-eze mice (**Fig. 3D**). *De novo* cholesterol synthesis, as measured from the appearance of labelled cholesterol in the plasma, was below the detection limit of 1% in both groups and could therefore not be used for reliable calculations.

Transintestinal cholesterol excretion after ezetimibe exposure

The cholesterol present in the intestinal lumen originates from the diet, from biliary secretion, and from trans-intestinal cholesterol excretion (TICE) [173]. The intestinal cholesterol balance can be estimated based on the net cholesterol transport across the intestine: the difference between fecal NS excretion and the sum of dietary and biliary cholesterol influx into the intestine (**Fig. 3E**). The amount of cholesterol secreted via the feces did not exceed the dietary and biliary influx, indicating that absolute cholesterol absorption may exceed TICE (**Fig. 3F-G**).

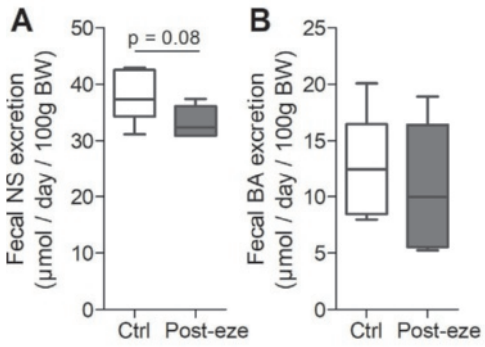

Figure 2: Fecal cholesterol and bile acid excretion two weeks after ezetimibe exposure. (A) Fecal NS excretion and **(B)** BA excretion at the age of 8 weeks, two weeks after the intervention diet with added ezetimibe.

Bile acids after ezetimibe exposure

At 6 weeks after ezetimibe intake, total plasma BA levels were not affected by the ezetimibe treatment early in life, neither was the bile flow (**Fig. 4A**; **Fig. 4B**, p = 0.14). BA secretion in the bile as well as BA excretion in the feces appeared unaffected (**Fig. 4C**, **4E**). The composition of fecal BA was virtually identical between the groups (data not shown), as depicted by the equal hydrophobicity index (**Fig. 4D**).

Gene expression after ezetimibe exposure

To assess the possible effect of early life ezetimibe exposure on expression of genes related to cholesterol homeostasis we measured relative gene expression levels in the proximal ileum (**Fig. 5**). *Npc1l1* expression appeared to be unaffected by post-

lactation ezetimibe treatment. *Hmgcr*, the gene encoding the rate-limiting enzyme of cholesterol synthesis, was also not affected. The protein encoded by *Abcg8* facilitates transport of sterols into the bile or back into the intestinal lumen. *Abcg8* did not differ between the groups, neither did *Abca1*, encoding for a cholesterol efflux transporter. *Srebp2*, required for lipid homeostasis and *Ldlr*, the low density lipoprotein receptor were expressed at equal level in control and post-ezetimibe treated mice.

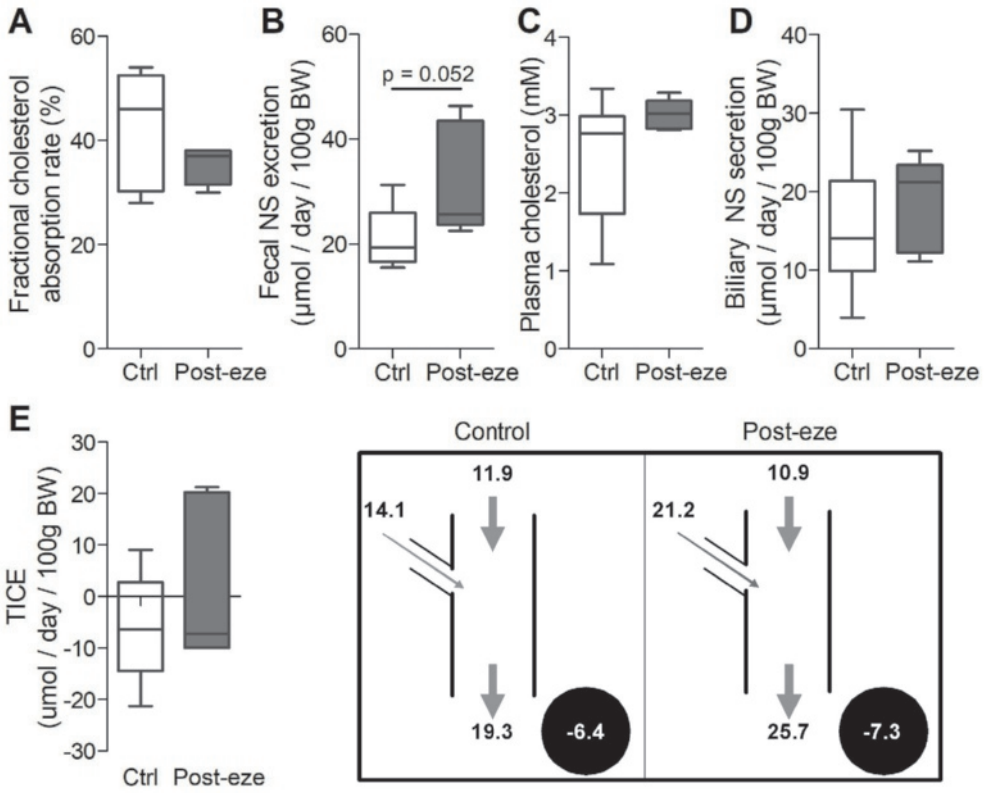

Figure 3: Cholesterol metabolism at six weeks after ezetimibe exposure. Parameters at 11-12 weeks of age, six weeks after the intervention diet. **(A)** Fractional cholesterol absorption. **(B)** Fecal NS excretion. **(C)** Plasma cholesterol. **(D)** Biliary cholesterol secretion. **(E)** Intestinal cholesterol balance = fecal NS excretion – (cholesterol intake + biliary NS). **(F)** Intestinal balance. **(G)** Schematic representation of intestinal net cholesterol and fecal neutral sterol fluxes (median values in µmol/day/100g BW).

Discussion

We investigated whether the sensitive window to program long-term the intestinal absorption efficacy of cholesterol by ezetimibe was extended beyond the lactation period in mice. Our results, however, indicate that there is no metabolic memory of ezetimibe treatment during a three weeks post-lactation period, whereas ezetimibe during the lactation period epigenetically lowered adult cholesterol absorption [59].

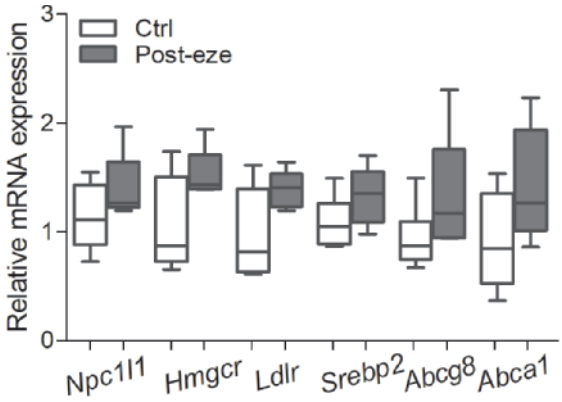

Figure 4: Bile acid parameters six weeks post-programming diet. Bile acid parameters in control and post-ezetimibe mice six weeks after ezetimibe administration. **(A)** Total bile acid concentration in plasma. **(B)** Hepatic bile flow. **(C)** BA secretion in hepatic bile. **(D)** Hydrophobicity index of bile. **(E)** Fecal BA excretion.

Upon administration immediately after weaning, ezetimibe significantly decreased cholesterol absorption with a corresponding increase in fecal NS excretion, similar to earlier reports in adolescent to adult male mice [50, 54, 86, 166, 174] and humans [48, 166, 175]. Our data indicate that within 6 weeks after the last ezetimibe administration, the effects on cholesterol absorption have almost completely disappeared: the fractional cholesterol absorption, plasma cholesterol levels and lipoprotein profiles are similar between the previously ezetimibe-receiving group and the control group. The only difference that we observed at 6 weeks after treatment (but not at 2 weeks after treatment) was a trend for increased fecal NS excretion in the previously ezetimibe-treated group. This suggests that although fractional cholesterol absorption and intestinal *Npc1l1* expression were not affected, ezetimibe may have shaped long-term cholesterol homeostasis. Ezetimibe is capable of changing the gut microbiota composition, specifically it may increase *Lactobacillus spp.* [176]. Increased abundance of specific *Lactobacillus spp*. has been linked to lower serum cholesterol [177], elevated BSH-activity in the feces [178], increased fecal cholesterol and BA excretion [87, 177, 179], and hepatic and intestinal expression of cholesterol homeostasis genes [176]. Thus, although the sensitive window for programming cholesterol absorption does not extend beyond

lactation, the data indicates some long-term effects on (microbiota)metabolism might occur.

Cholesterol disposal from the body occurs quantitatively by direct secretion of cholesterol into the intestine or via hepatic conversion of cholesterol into bile acids and subsequent excretion into the feces [170]. Interestingly, fecal BA excretion was decreased during ezetimibe treatment ('acute' effect) and similar to the control group 2 and 6 weeks after treatment. This acute effect of ezetimibe administration was not reported in earlier studies in mice and humans in which ezetimibe either increased or did not affect fecal BA excretion [166, 174, 175, 180]. The observed decrease in fecal BA could be attributed to reduced levels of α- and β-muricholic acids. Alpha- and β-muricholic acids are considerably hydrophilic and have been characterized as FXR antagonists [93]. Thereby, muricholic acids can inhibit cholesterol absorption and stimulate BA synthesis [94, 168, 169]. It is difficult to explain the observed decrease in fecal BA with the current knowledge and data.

Our data seem to support the hypothesis by Noah *et al.*, stating that murine intestinal development is completed at weaning [171]. During lactation intestinal cells still develop, including the intestinal stem cells which give rise to the enterocytes for the rest of the lifetime. Enterocytes have an absorptive function, including absorption and further metabolism of cholesterol [81, 171]. During lactation, the intestinal stem cells are under influence of many epigenetic changes which may affect long-term intestinal stem cell function and consequent metabolic health [81]. However, other studies indicate changes in enterocytes upon weaning due to the transition from fluid to solid nutrition [172].

Our data do not provide indications that manipulation of intestinal cholesterol absorption beyond the murine lactation period affects long-term cholesterol

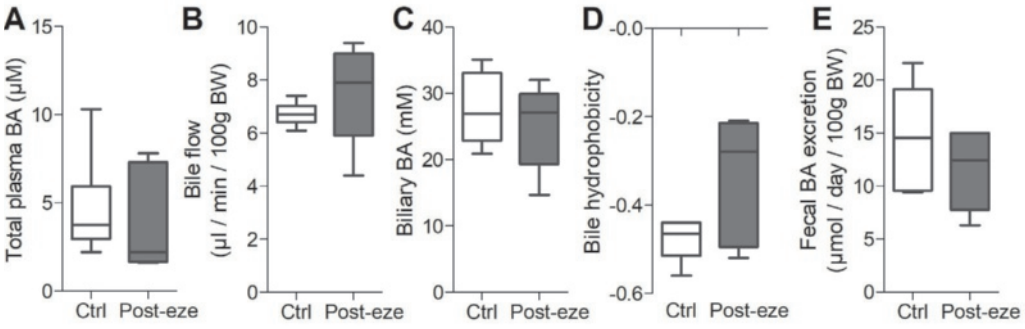

Figure 5: Intestinal gene expression levels six weeks post-programming diet. mRNA expression levels of cholesterol transport-related genes in the proximal ileum of control and post-ezetimibe treated mice at the age of 12 weeks, relative to *36B4*.

absorption. It remains to be determined whether the "shutting off" of the sensitive window is related to age or development or, rather to the switch from milk to solid food. If the latter is important, it may be possible that prolongation of the lactation period such as physiological in the human situation, may still impose a window of opportunity for programming. To explore the dynamics of the window of intestinal plasticity, the epigenetic changes in intestinal stem cells could be determined in a study with different pre- and post-lactation duration of liquid nutrition similar to breast milk composition. If long-term programming of cholesterol homeostasis in early life is linked to epigenetic modification of the *Npc1l1* gene, translation of murine data should take into account that the *Npc1l1* expression pattern is different in humans. Human *NPC1L1* is not only expressed in the small intestine but also in the liver, where the protein reduces biliary cholesterol secretion [181]. Therefore, ezetimibe in humans increases endogenous cholesterol excretion via the bile [48] and may thus have a different long-term outcome.

Apart from the time of ezetimibe administration, several differences should be taken into account when comparing this study to our previous work (2017) [59]. First, we used males instead of females. Research shows that there are many sex differences in metabolism and also with respect to the risk of disease due to early life events [13, 99, 182]. In adult humans, ezetimibe treatment with or without statin achieves better lipid-lowering effects in men than in women [183, 184]. This difference is possibly related to genetic variations in the *NPC1L1* gene [185]. Second, instead of LDL-receptor knockout mice we used wild-type C57BL/6JOlaHsd mice whose lipoprotein profile is more distinct from humans. Yet, cholesterol exposure via maternal high-cholesterol diet during (pre-)gestation and lactation can program lipoprotein parameters in C57BL/6J mouse offspring up into adulthood [186, 187], indicating that nutritional cues such as cholesterol in early life does have the ability to program metabolism in this strain.

Taken together, our data suggests that the sensitive window for programming adult cholesterol absorption by ezetimibe does not extend beyond lactation in male mice, although ezetimibe does affect cholesterol disposal possibly via the microbiota. If the same is true for humans, adapting long-term cholesterol absorption to improve cardiovascular health should occur before the end of lactation.

Materials & Methods

Animal studies. We used C57BL/6JOlaHsd mice (Envigo) which were group-housed in temperature-controlled conditions with 12:12 light dark cycles. For breeding, males were

kept on AIN-93M+0.15% cholesterol and females on AIN-93G+0.15% cholesterol diet (Research Diets, New Brunswick, USA). Nests were standardized to n = 6 with 4 males + 2 females on postnatal day 1-3. Offspring was weaned into individual cages and divided into two dietary groups for three weeks: AIN-93G+0.15% cholesterol (control, n = 6) and control + 0.005% Ezetimibe (Ezetrol (R); Eze, n = 5). Cholesterol absorption was measured at 5-6 weeks of age. Feces were collected at 5, 8 and 11 weeks of age. From six to 12 weeks of age all mice received AIN-93M+0.11% cholesterol (Research Diets, New Brunswick, USA). We repeatedly measured body weight and at 6 and 11 weeks of age body composition analysis was performed by nuclear magnetic resonance (NMR) for small animals (Minispec LF90 Body Composition Analyzer; Bruker, Germany). At age 11-12 weeks, we assessed the following cholesterol homeostasis parameters: dietary cholesterol intake, intestinal absorption, *de novo* synthesis, biliary secretion rate, and finally, bile composition and fecal excretion rate. Gallbladder cannulation was performed at 12 weeks to collect hepatic bile as described previously [154]. Briefly, bile was cannulated for 20 minutes under Hypnorm (fentanyl/fluanisone; 1 mL/kg) and diazepam (10 mg/kg) anesthesia using a humidified incubator to maintain body temperature. After cannulation, blood was obtained via heart puncture, the mice were sacrificed and adipose tissue, liver and intestine were excised and snap-frozen in liquid nitrogen. All animal experiments were approved by the ethical committee for animal experimentation (IACUC) at the University of Groningen and performed in accordance with the Dutch National Law on Animal Experimentation and international guidelines on animal experimentation. A schematic of the detailed set-up of the study is shown in **Figure 1A**.

Fractional Intestinal Cholesterol Absorption. Fractional cholesterol absorption was measured using the plasma dual-isotope ratio method as described previously [59]. Briefly, at the end of the dark phase the non-fasted 10.5-week-old animals were injected intravenously with 0.3 mg ^{5}D-cholesterol dissolved in Intralipid (20%; Fresenius Kabi, Den Bosch, The Netherlands) and orally gavaged with 0.6 mg ^{7}D-cholesterol dissolved in medium-chain triglyceride oil. Blood spots from the tail were collected on filter paper before and after administration of the isotopes at 3, 6, 12, and 24 hours for the first day, and after that every 24 hours for the next consecutive 7 days. Cholesterol was extracted from blood spots, followed by analysis by GC-MS. Briefly, the calculation of fractional cholesterol absorption was based on the decay curves of ^{5}D (intravenously) and ^{7}D-cholesterol (oral) in plasma after their correction with the administered dose: F*a* = (area under the label enrichment curve curve$_{oral}$/area under the label enrichment curve curve$_{intravenous}$ × dose$_{intravenous}$/dose$_{oral}$) × 100.

Cholesterol synthesis and balance. Fractional cholesterol synthesis was determined by Mass Isotopomer Distribution Analysis (MIDA) using 13C-acetate (Isotec, Miamisburg, OH,

USA) as labeled precursor as described previously [159]. Transintestinal cholesterol excretion (TICE) was calculated as dietary cholesterol intake + biliary cholesterol secretion – fecal neutral sterol excretion.

Measurement of cholesterol, lipoprotein profiles and bile acids in plasma and bile. Total plasma cholesterol was measured enzymatically using a commercially available kit (Roche Diagnostics GmbH, Mannheim, Germany). Lipoprotein fractions of pooled plasma samples (n = 5-6) were separated via fast protein liquid chromatography (FPLC) gel filtration using a superose 6 column (GE Healthcare, Little Chalfont, UK) as published [Wiersma 2009]. Samples were chromatographed at a flow rate of 0.5 mL/min, and lipoprotein fractions of 500 µl each were collected. Individual fractions were assayed for cholesterol concentrations as described above. Biliary and plasma bile acid (BA) concentrations were determined using liquid-chromatography mass spectrometry (LCMS) as described previously [188]. Neutral sterols (NS) were extracted from bile according to Bligh and Dyer [153] followed by derivatization in BSTFA, pyridine, and TMCS (5:5:0.1), re-dissolving in heptane with 1% BSTFA, and measurement by GC. Total bile acids from bile were measured using an enzymatic fluorescent assay according to Mashige et al. [189].

Cecum analysis. Bacterial DNA of the available cecum content was isolated, subsequently measured using MiSeq sequencing of the amplified 16S rRNA genes and analyzed by the QIIME and ARB method as described in Heida *et al.* [190].

Fecal neutral sterols and bile acids. Fecal samples from individually housed mice collected over 24 hours were dried, weighed and ground to powder. NS and BA profiles were determined using gas-liquid chromatography as published [154, 155]. Briefly, 50 mg feces was saponified in the presence of 1 mL alkaline methanol (1:3 NaOH:methanol) by heating for 2 hours at 80°C. The NS were extracted with petroleum ether, derivatized in a mixture of N,O-bis-trifluoroacetamide (BSTFA), pyridine, and trimethylchlorosilance (TMCS) in a ratio of 5:5:0.1, re-dissolved in heptane containing 1% BSTFA, and measured by gas chromatography (GC). After NS extraction, total bile acids were extracted from the aqueous phase using SepPak C18 cartridges (Waters, Dublin, Ireland), methylated, and after derivatization with BSTFA, pyridine, and TMCS, were measured by GC. The same methodology was applied for the determination of cholesterol content using a 50 mg aliquot of the animal food; by knowing the food intake, the dietary cholesterol intake could be calculated.

Gene expression. Proximal duodenum mRNA was extracted using TriReagent (Sigma) and quantified with a Nanodrop ND-100UV-vis spectrometer (NanoDrop Technologies Wilmington DE). cDNA was made from 1µg of RNA using reagents from Invitrogen

(Carlsbad CA). Primers were synthesized by Eurogentec (Seraing, Belgium). Real-time PCR was performed using an ABI Prism 7700 machine (Applied Biosystems, Damstadt Germany). mRNA expression levels of individual genes were calculated relative to the housekeeping gene *36b4*.

Statistical analysis. The statistical analysis was performed with GraphPad Prism 5 Software using Mann-Whitney U Test. P-values below 0.05 were considered significant (* $p < 0.05$; ** $p < 0.01$). Graphs are made with GraphPad Prism 5; using the Tukey method for plotting the box ((25^{th} - 75^{th} percentiles), whiskers (1.5 IQR) and outliers (>1.5 IQR) unless stated otherwise. Open symbols = control group (n = 6); grey symbols = post-ezetimibe group (n = 5).

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Author contributions

Testing, data acquisition and analysis, and drafting the article were performed by M.A.M.L.. U.J.F.T. and H.J.V. were responsible for the conception, design, and supervision of the study, interpretation of data, and critical article revision for important intellectual content. All contributing authors gave final approval for the version to be published.

Additional information

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Competing interests.

The authors declare that they have no competing interests.